RESEARCH ARTICLE

Quantitative Real-Time RT-PCR of *ITGA7, SVEP1, TNS1, LPHN3, SEMA3G, KLB* and *MMP13* mRNA Expression in Breast Cancer

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Abstract

Breast cancer is the leading cause of cancer deaths among women worldwide, including Thailand. In the present study, the differential mRNA expression of *SVEP1, LPHN3, KLB, ITGA7, SEMA3G, TNS1* and *MMP13* genes was examined in breast cancer using quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR). Among these genes, increased *LPHN3* and *MMP13* mRNA expression levels correlated with axillary-node metastasis (P=0.02). Multiple logistic regression analysis revealed that *LPHN3* and *MMP13* mRNA expression is significantly associated with axillary node status in breast cancer (P=0.04).

Keywords: *LPHN3 - MMP13* - breast cancer - lymph node metastasis

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Introduction

Breast cancer is the most diagnosed cancer among women worldwide, with an incidence of 39.0/100,000/year and a death rate of 12.5/100,000/year (Ferlay et al., 2008). In Thailand, breast cancer is the most common cancer among women. The national incidence of breast cancer is 30.7/100,000/year and the death rate is 10.8/100,000/year (Ferlay et al., 2008).

Aberrant expression in several genes is reportedly a risk factor in both familial and sporadic breast cancer. The most common genetic alterations in familial breast cancer occur in *BRCA1, BRCA2, CHEK2, TP53,* and *PTEN*, whereas alterations in *MYC, CCND1*, and *ERBB2* play a role in sporadic breast cancer (Kenemans et al., 2008). Among these genetic alterations, overexpression of *ERBB2 (HER2/neu)* was associated with clinical outcome of breast cancer, and was used as a target for breast-cancer therapy (Yu et al., 2000). However, the molecular basis of sporadic breast cancer remains inconclusive. A recent study of the gene expression profiles of Thai sporadic-breast-cancer patients found that 928 genes were expressed differentially (unpublished observations). In this study, the differential expression of 7 mRNA - *SVEP1* (Sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1), *LPHN3* (Latrophilin 3), *KLB* (Klotho beta), *ITGA7* (Integrin, alpha 7), *SEMA3G* (Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3G), *TNS1* (Tensin 1), and *MMP13* (matrix metallopeptidase 13) were analyzed by quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR). These genes play a role in cell adhesion and migration (Elnemr et al., 2003; Shur et al., 2007; Karayan-Tapon et al., 2008; Hall et al., 2009; Zhu et al., 2010; Martinez et al., 2011; Yang et al., 2012). Moreover, the association between mRNA expression and the clinical characteristics of these patients was evaluated.

Materials and Methods

Patients and tissue samples

The breast-cancer tissues and corresponding adjacent normal tissues of 33 patients were obtained from the Pathology Division, Army Institute of Pathology, Phramongkutklao Medical Center, Bangkok, Thailand, in the period 2006-2010. After resection, all specimens were immediately stored in TRIzol® reagent (Invitrogen, USA) at -80°C until use. No patient had undergone chemotherapy before the operation. Each hematoxilin and eosin-stained tumor sample was examined microscopically by a pathologist. Tissues with >90% tumor cells were used in this study. This study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (MUTM 2011-012-02).

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted using TRIzol® reagent (Invitrogen, USA) according to the manufacturer's instructions. The quantity of RNA was measured by Nanodrop 1000 spectrophotometer (Thermo Fisher

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Scientific, USA) and its quality assessed by visualizing the 28S and 18S rRNA bands using 1.5% gel electrophoresis. Reverse transcription of mRNA was performed using Superscript® VILO™ cDNA Synthesis kits (Invitrogen, USA), according to the manufacturer's instructions. cDNA was then purified using a DNAclear™ cDNA Purification Kit (Applied Biosystems, USA). The concentration of purified cDNA was determined by Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). To determine cDNA quality, conventional PCR was used with *β-actin* primer. For QRT-PCR, specific primers of 7 genes, including *β-actin* primer, were designed by Primer-BLAST program (NCBI) using nucleotide sequences from the NCBI database (Table 1). The reaction was performed in 20 μl of reaction mixture containing 2 μl of 10X LightCycler® FastStart DNA Master SYBR Green I (Roche, USA), 1 μl of 5 μM forward primer, 1 μl of 5 μM reward primer, 1.6 μl of 25 mM of $MgCl_2$, 12.4 μl of sterile distilled water and 2 μl of 10 ng cDNA per reaction. Amplification was performed in a LightCycler® Real-Time PCR system (Roche Applied Science, USA). The first step consists of pre-denaturation at 95°C for 2 minutes; the second 45 cycles of denaturation at 95°C for 30 seconds; annealing temperature dependent upon primer, and extension at 72°C for 1 minute. A *β-actin* gene was used as the reference control gene. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative change in gene expression determined by real-time PCR, according to a previous study (Nan et al., 2006). Increased mRNA expression was defined as N-fold change ≥2, normal mRNA expression was defined as N-fold change ranging between 0.5-2, and decreased mRNA expression was defined as N-fold change ≤0.5. All samples were run in duplicate.

Statistical analysis

Categorical data were expressed as frequency (percentage) and continuous data as mean±SD. Association between mRNA expression patterns and clinicopathological parameter-age, tumor size, axillary node status, triple negative breast cancer (TNBC) status-were analyzed by univariate logistic regression and multivariate logistic regression. Statistical analysis was performed using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA), and a P value of < 0.05 was considered statistically significant.

Table 1. Location, Nucleotide Sequences and Amplicon Sizes of Specific Primers for QRT-PCR Analysis

Gene	Location	Primer sequence (5'-3')	Product size (bp)
β-actin	7p22	F: TCACCCACACTGTGCCCATCTACGA	295
		R:CAGCGGAACCGCTCATTGCCAATGG	
TNS1	2q35-q36	F: TCAAGTGGAAGAACTTGTTTGCTT	86
		R: CACGACAATATAGTGGAGGCACA	
KLB	4p14	F: AACTTACAACACATACCATTAA	249
		R: GTTTACATCCAAGAACTGAGT	
LPHN3	4q13.1	F: ACATATCAAGCAGTCAGAGGAA	150
		R: GTTAGGTGGTTACAAGAGCATGT	
SVEP1	9q32	F: GTTGCATTGAGGAGTTAGCATT	106
		R: CTGTTAGCAAGACAGGATGATT	
ITGA7	12q13	F: GCTGTGAAGTCCCTGGAAGTGATT	80
		R: GCATCTCGGAGCATCAAGTTCTT	
SEMA3G	3p21.1	F: TGTATGCTATTAACTCCTGGAA	237
		R: ACAATACACAGATACACAGTAAT	
MMP13	11q22.3	F: TCGCGTCATGCCAGCAAATTCCAT	116
		R: TTCTTCCCCTACCCCGCACTTCTG	

F=forward, *R*=reverse, b*p*=base pair

Results

The clinical characteristics of the patients and the expression profiles of 7 mRNA are shown in Table 2. All patients had ductal-type carcinomas. The mean age of the

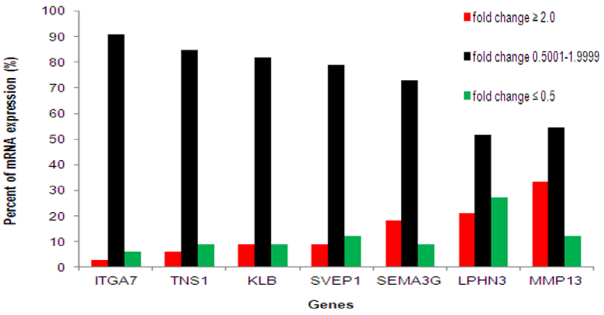

Figure 1. Differential Gene Expression Frequency in Breast Cancer. Expression patterns of *ITGA7*, *SVEP1*, *TNS1*, *LPHN3*, *SEMA3G*, *KLB* and *MMP13* mRNA in breast-cancer tissues were analysed by QRT-PCR using *β-actin* mRNA as a control. The bar graph presents percent mRNA expression in breast-cancer patients.

Table 2. Clinicopathological Parameters and mRNA Expression Patterns of 7 Genes of Breast Cancer Patients

No.	Age	Tumor size (cm)	Axillary node	TNBC*	mRNA expression patterns**						
					LPHN3	*MMP13*	*ITGA7*	*SVEP1*	*TNS1*	*KLB*	*SEMA3G*
1	76	3	6/8	-	-	-	-	-	-	-	-
2	80	1.5	1/12	-	n	-	-	-	-	-	-
3	70	2.7	16/16	-	+	+	-	n	-	n	-
4	53	2	0/2	-	n	+	-	-	-	+	+
5	54	2.5	3/23	-	+	-	-	+	n	-	-
6	83	3.5	2/8	-	+	-	-	-	-	-	-
7	37	3	0/1	-	-	+	+	+	+	+	+
8	91	1.7	0/14	-	-	-	-	-	-	-	-
9	43	2.5	5/30	-	n	+	-	-	-	-	-
10	41	2.5	1/1	-	n	+	-	n	n	+	+
11	38	1	15/21	+	+	-	-	n	-	-	-
12	68	3	0/13	+	-	-	-	+	-	-	-
13	54	3.5	0/1	-	-	n	-	-	-	-	-
14	48	2.5	2/19	+	n	-	-	-	-	-	-
15	59	4	1/1	-	-	-	-	-	-	-	-
16	44	2.8	0/19	-	-	-	-	-	-	-	-
17	65	2.5	0/13	-	-	-	-	-	-	-	-
18	51	2	0/1	-	-	-	-	-	-	-	-
19	71	1.5	0/2	-	n	-	-	-	-	-	n
20	39	2	0/3	+	n	-	n	n	+	-	n
21	45	2	7/23	-	+	+	n	-	n	n	+
22	66	2.5	7/15	-	+	-	-	-	-	-	n
23	58	4.5	3/9	-	n	+	-	-	-	-	+
24	78	3.5	0/3	+	-	-	-	-	-	-	-
25	39	1.5	0/1	-	-	+	-	-	-	-	-
26	67	3	1/13	-	-	+	-	-	-	-	-
27	54	2.5	1/16	-	n	+	-	-	-	-	-
28	46	2	0/3	+	-	+	-	-	-	-	+
29	69	2.5	0/1	-	-	-	-	-	-	-	-
30	34	6	5/17	-	-	-	-	-	-	-	-
31	59	3.2	0/19	-	+	n	-	-	-	-	-
32	61	1	0/1	-	-	n	-	-	-	-	-
33	60	6	1/6	-	-	+	-	-	-	-	-

*TNBC= triple-negative breast cancer (Estrogen receptor, Progesterone receptor, Human epidermal growth factor receptor 2) (+ triple negative, - non-triple negative), *mRNA expression patterns (+=increased, -=decreased, n=normal)

Table 3. Univariate Logistic Regression Analysis of *LPHN3* and *MMP13* mRNA Expression and Clinicopathological Parameters in Breast Cancer

Parameters		Increased mRNA expression Yes n (%)	No n (%)	OR (95%CI)	*P* value
Age at diagnosis	≤50	7 (64)	4 (36)	2.1 (0.47-9.3)	0.32
	>50	10 (45)	12 (55)		
Size (cm)	≤3	13 (52)	12 (48)	1.08 (0.22-5.33)	0.92
	>3	4 (50)	4 (50)		
Axillary node status	-	5 (31)	11 (69)	5.28 (1.20-23.3)	0.02*
	+	12 (71)	5 (29)		
TNBC	-	15 (56)	12 (44)	0.4 (0.06-2.57)	0.33
	+	2 (33)	4 (67)		

**OR* odds ratio, *CI* confidence interval, Axillary node status (-no node metastasis, + node metastasis), *TNBC*=triple-negative breast cancer (+triple-negative, -non-triple negative), *Statistically significant correlation (P<0.05)

Table 4. Multivariate Logistic Regression Analysis of *LPHN3* and *MMP13* mRNA Expression

Factors	OR (95%CI)	*P* value
Age range, ≤50 versus >50 years	3.27 (0.47-22.7)	0.23
Tumor size, ≤3 versus >3 cm	1.18 (0.17-8.02)	0.86
Axillary node status, no node versus node metastasis	4.98 (1.04-23.9)	0.04*
TNBC, non-triple-negative versus triple-negative	0.27 (0.02-3.0)	0.28

*Statistically significant correlation (P<0.05))

patients was 58±15 years (range: 34-91). Eleven patients (33%) were aged ≤50 years and 22 (67%) >50 years. Twenty-five patients (76%) had tumors ≤3.0 centimeters (cm) in size, and 8 (24%) had tumors >3.0 cm. Seventeen patients (52%) were axillary-node-positive and 16 (49%) -negative. Twenty-seven patients (82%) were non-triple negative breast cancer and 6 (18%) - triple negative breast cancer.

QRT-PCR was conducted to determine the mRNA expression of these 7 genes in 33 breast cancers and their adjacent corresponding normal tissues, using the *β-actin* gene as reference control. The distributions of expression for the 7 genes are shown in Figure 1. The mRNA expression patterns of these 7 genes were analysed further in conjunction with the patients' clinicopathological parameters. The results showed that increased mRNA expression of *LPHN3* and *MMP13* correlated statistically with axillary node status (P=0.02, OR=5.28, 95%CI=1.20-23.3) (Table 3).

The results of multivariate logistic regression analysis for the relationship between increased *LPHN3* and *MMP13* mRNA expression level and clinicopathological variables are presented in Table 4. Increased mRNA expression of *LPHN3* and *MMP13* was significantly associated with axillary node metastasis (P=0.04, OR=4.98, 95%CI=1.04-23.9).

Discussion

In breast cancer, the most important prognostic factors are nodal status, tumor size, hormone receptor (HR) status, and histological grade (Fisher et al., 1995), whereas other clinicopathological factors and novel molecular markers are under investigation to improve the predictability of clinical outcome. One of these factors, axillary lymph node status, is important for determining the risk category for breast cancer. The accuracy of lymph-node evaluation influences clinicians' choice of the appropriate therapeutic regimen and most likely prognosis (Fisher et al., 1983; Jatoi et al.,1999; Yang et al ., 2012). In recent years, many sensitive methods have been developed to detect micro-metastasis in lymph nodes, e.g., immuno-histochemical (Cabioglu et al., 2005; Denninghoff et al.,2008; Thongwatchara et al., 2011; Yenidunya et al.,2011) and molecular biological techniques. However, the role of micro-metastasis in clinical outcome is under extensive study.

Several studies have suggested that using QRT-PCR, panel gene marker (*STC, GalNacT,* and *MAGE-A3*) expression in circulating tumor cells can distinguish sentinel lymph-node (SLN) and non-SLN metastasis with sensitivities of 96, 88, and 68%, respectively (Nakagawa et al., 2007). Moreover, the expression of *CK19, MGB1, MGB2, CEA, EPCAM,* and *NY-BR-1* was found sensitive for detecting SLN metastasis (95, 82, 82, 72, 92 and 82%, respectively) compared with histology (Wallwiener et al., 2011). *CK19, DNMT3b,* and *MMG* gene expression, when analyzed by QRT-PCR, were found to be relatively sensitive in detecting axillary node metastasis (96, 88, and 68%, respectively), concurring with histological features (Berger et al., 2006).

This study found that increased mRNA expression of *LPHN3* and *MMP13* in breast-cancer tissues significantly correlated with axillary lymph node positivity, which was particularly interesting. *LPHN3* (the latrophilin 3 gene) is a member of the G-protein coupled receptor (GPCR) family; it has large extracellular and intracellular domains, containing several cell adhesion modules, such as cadherin, IgG, laminin A, thrombospondin type 1, galactose lectin, EGF, and transmembrane segments that may be involved in intracellular signalling during cell-to-cell adhesion (Martinez et al., 2011). *LPHN3* was up-regulated significantly in a transgenic mice model that over-expressed myocilin (Paper et al., 2008). Altered *LPHN3* expression levels have been seen after brain ischemia (Bin et al., 2002), and during the period of adrenal gland development (Wing et al., 2009). *LPHN3*-deficient mice display evidence of profound disruption at multiple monoamine-signalling levels, causing attention deficit-hyperactivity disorder (ADHD), the most common psychiatric disorder in childhood and adolescence (Wallis et al., 2012). *MMP13* or collagenase-3 is a member of the matrix metalloproteinase family of endopeptidases that have potent degrading activity for degrading the major protein components of the extracellular matrix and basement membranes of the cells (Pendás et al., 2000). *MMP13* is induced during invasion and metastasis of head and neck cancer, skin cancer, gastric cancer and breast cancer (Nielsen et al., 2001; Elnemr et al.,2003; Luukkaa et al., 2006; Lederle et al., 2010). This study demonstrated that mRNA expression of *LPHN3* and *MMP13* is directly linked to tumor aggressiveness, since the common first route of spread for breast carcinoma is through the axillary lymph nodes. This process may be modulated by the expression of *LPHN3* and *MMP13*.

Increased mRNA expression of *LPHN3* and *MMP13* could be a marker associated with breast cancer metastasis in the axillary lymph nodes, which may become a clinical marker for predicting axillary node status accurately. The role of *LPHN3* and *MMP13* in axillary-node metastasis in breast cancer deserves further study.

In summary, increased *LPHN3* and *MMP13* mRNA expression is significantly associated with axillary node status in breast cancer, which may help improve the planning of appropriate chemotherapeutic treatment. However, larger sample sizes should elicit more precise data.

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